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Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants

Antje von Schaewen, Mark Stitt[1], Renate Schmidt, Uwe Sonnewald and Lothar Willmitzer

Institut für Genbiologische Forschung Berlin GmbH, Ihnestrasse 63, 1000 Berlin 33 and [1]Institut für Pflanzenphysiologie, Universität Bayreuth, 8580 Bayreuth, FRG

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Chimeric genes consisting of the coding sequence of the yeast invertase gene *suc 2* and different N-terminal portions of the potato-derived vacuolar protein proteinase inhibitor II fused to the 35S CaMV promoter and the poly-A site of the octopine synthase gene were transferred into tobacco and *Arabidopsis thaliana* plants using *Agrobacterium* based systems. Regenerated transgenic plants display a 50- to 500-fold higher invertase activity compared to non-transformed control plants. This invertase is N-glycosylated and efficiently secreted from the plant cell leading to its apoplastic location. Whereas expression of the invertase does not lead to drastic changes in transgenic *Arabidopsis thaliana* plants, transgenic tobacco plants show dramatic changes with respect to development and phenotype. Expression of the invertase leads to stunted growth due to reduction of internodal distances, to development of bleached and/or necrotic regions in older leaves and to suppressed root formation. In mature leaves, high levels of soluble sugars and starch accumulate. These carbohydrates do not show a diurnal turnover. The accumulation of carbohydrate is accompanied by an inhibition of photosynthesis, and in tobacco, by an increase in the rate of respiration. Measurements in bleached versus green areas of the same leaf show that the bleached section contains high levels of carbohydrates and has lower photosynthesis and higher respiration than green sections. It is concluded that expression of invertase in the cell wall interrupts export and leads to an accumulation of carbohydrates and inhibition of photosynthesis.

Key words: invertase/photosynthesis/source–sink transition/transgenic plant

Introduction

Growth and development of a plant are dependent upon the energy gained by fixing carbon dioxide into carbohydrates during photosynthesis. Primary sites for photosynthesis are leaf and to a much lesser extent stem tissues, whereas other organs such as roots, seeds or tubers do not contribute to carbon assimilation but rather totally depend for their fixed carbon dioxide on photosynthetically active organs. Thus there is a net flow of energy from photosynthetically active tissues, representing the sources (defined as net exporters of fixed carbon) to photosynthetically inactive parts of the plant, representing the sinks (defined as net importers of fixed carbon).

Plant development is accompanied by continuous changes with respect to an organ representing a sink or a source. Essentially all plant organs act as a sink at some stage. During plant development, however, the relative sink strength (defined as the ability of a sink organ to import assimilates) changes (Ho, 1988). During vegetative growth, roots and developing leaves constitute strong sinks, whereas with the onset of flowering the bulk of the assimilate flows into developing seeds, roots or tubers, which therefore represent the strongest sinks during this developmental stage. Sink–source relationships are therefore not only central with respect to crop yield but also represent a fascinating biological system closely linked to plant development (for review see Turgeon, 1989).

Several models have been described in the literature dealing with the central problem of sink–source transitions as well as with the question of what factors determine sink strength and source capacity. One parameter important for the source capacity of a leaf is its photosynthetic activity. It has been suggested that carbohydrate accumulates in the leaf and photosynthesis is inhibited when the rate of photosynthesis exceeds the demand for sucrose in the sinks (Neales and Incoll, 1968; Geiger, 1976; Herold, 1980). This 'feed-back' or 'sink' regulation of photosynthesis has been studied by inhibiting phloem transport e.g. by cold-girdling or detaching leaves (Aczon-Beito, 1983; Rufty and Huber, 1983; Blechschmidt-Schneider *et al.*, 1989; Shaw *et al.*, 1986) or by altering the sink–source balance in whole plants. The latter can be achieved in several ways e.g. by removing potential sinks (Claussen and Biller, 1976; Fondy and Geiger, 1980; Shaw *et al.*, 1986), inhibiting sink metabolism (Bagnall *et al.*, 1988), increasing the supply of light or CO_2 to the source leaf (Cave *et al.*, 1981; Nafziger and Koller, 1976), or by removing other competing source leaves (Clough *et al.*, 1981; Rufty and Huber, 1983; Sasek *et al.*, 1985).

Many of these studies found that photosynthesis was inhibited when sink demand was decreased. This inhibition of photosynthesis was often accompanied by an accumulation of carbohydrate (Neales and Incoll, 1968; Nafziger and Koller, 1976). However, this was not always the case (Neales and Incoll, 1968; Geiger, 1976; Clausen and Biller, 1976; Carmi and Shomer, 1979; Ntrika and Delrot, 1986), and none of these experiments established an unequivocal causal relationship between the accumulation of carbohydrate and the inhibition of photosynthesis. Many of these treatments were also rather unspecific, and would also affect the movement of nitrogen, minerals and hormones.

Sucrose is probably exported from the leaf by transferring it to the apoplast and then actively loading it into the phloem (Turgeon, 1989). It should be possible to inhibit sucrose export by introducing and/or overexpressing an invertase into

the cell wall. After entering the apoplast, sucrose would be hydrolysed to glucose and fructose. These free hexoses cannot be taken up into the phloem as well as sucrose (Kallarakal and Komor, 1989). Instead, they are retrieved by the mesophyll cells (Maynard and Lucas, 1982), rephosphorylated by hexokinase and fructokinase and then reconverted to sucrose. Furthermore, inhibiting the export of sucrose from source leaves would represent a rather severe change with respect to sink−source interactions and thus these plants should lead to valuable insights into principles governing this interaction.

Here we have taken advantage of the possibility to construct transgenic plants in order to create such a situation. To this end an invertase gene (*suc 2*) from *Saccharomyces cerevisiae* was used. The rationale for choosing the alien yeast invertase was to minimize the possibility that the enzyme would be inhibited by endogenous invertase inhibitors. Furthermore, yeast invertase is known to be active over a broad pH range (Goldstein and Lampen, 1976) which is important with respect to the fact that the pH of the apoplastic space of plants is not known precisely.

Yeast invertase occurs in a cytoplasmic and an extracellular (periplasmic) form, both being encoded by the same gene (Perlman and Halvorson, 1981). Targeting experiments performed in yeast have provided evidence that the protein is secreted by the default pathway unless it is linked to other protein moieties which specify vacuolar transport signals (Johnson *et al.*, 1987; Valls *et al.*, 1987). Thus to direct the yeast invertase into the cell wall, signal peptides derived from a vacuolar plant protein, i.e. the proteinase inhibitor II from potato (Keil *et al.*, 1986; Nelson and Ryan, 1980) were used.

As sink−source relationships are known to differ among different plant species, two different host plants were used for this experiment which differ widely in their growth habits, growth rates and generation times i.e. *Nicotiana tabacum* and *Arabidopsis thaliana*.

The data presented demonstrate that expression of an apoplastic invertase leads to decreased sucrose export, accumulation of carbohydrate and inhibition of photosynthesis which would be in agreement with photosynthesis being sink-limited although more complicated explanations cannot yet be excluded (see Discussion). Furthermore, in the case of tobacco, dramatic changes of development and phenotype of invertase-expressing plants were observed.

Results

Proteinase inhibitor II−invertase fusion proteins are glycosylated when in vitro translated in the presence of microsomal vesicles

As outlined in the Introduction, apoplastic invertase represents a prime candidate for interfering with the sink−source relationship by preventing sucrose export from source leaves.

To ensure uptake of yeast invertase into the endoplasmic reticulum of plant cells, N-terminal sequences derived from a vacuolar plant protein, the proteinase inhibitor II (PI-II) of potato (Keil *et al.*, 1986), were fused in front of the invertase gene encoding the mature protein, i.e. lacking its own signal peptide. As we did not know what to expect regarding the efficient uptake of the chimeric proteins by the ER, different constructs with N-terminal portions of PI-II differing in length were prepared. The shortest construct ended three amino acids after the PI-II signal peptide cleavage site (PI-3-INV), the middle construct contained an additional 55 amino acids (PI-58-INV) and the longest construct included an additional 110 amino acids (PI-113-INV) of the mature PI-II protein (123 amino acids) (see Figure 1 for a more detailed description of the different constructs).

As a first step, different PI-II−invertase fusions were tested with respect to their ability to enter the lumen of microsomal vesicles in an *in vitro* transcription/translation system. After removal of the introns, the constructs described in Figure 1 were inserted into SP6 expression vectors.

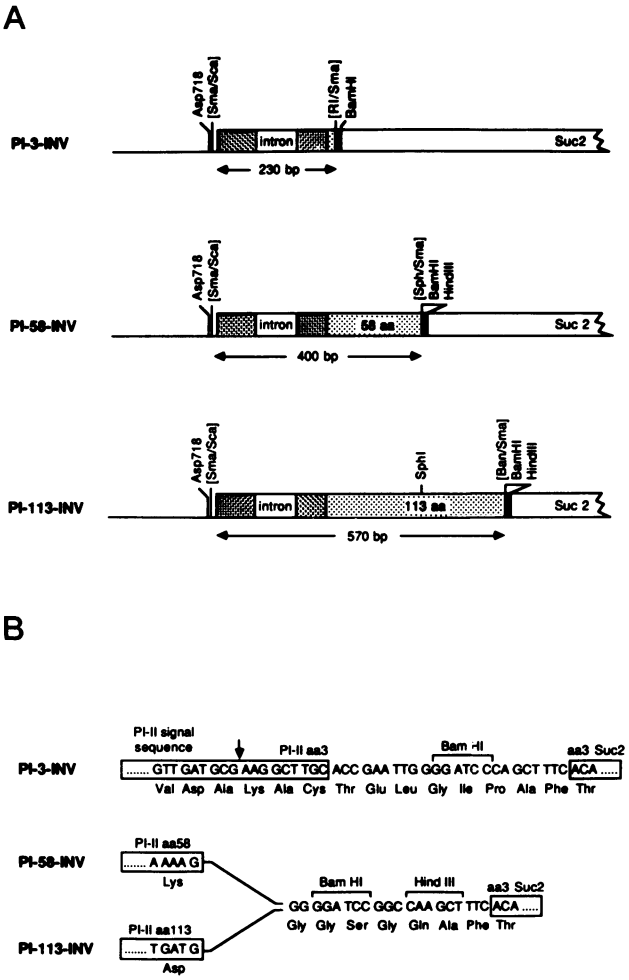

Fig. 1. Structure of the three fusion proteins made from the invertase gene (*suc 2*) from yeast and different N-terminal portions of the proteinase inhibitor II gene. Part **A** shows the schematic structure and location of restriction sites in three fusion proteins containing N-terminal portions of the proteinase inhibitor II gene from potato (Keil *et al.*, 1986) and the coding region of the invertase gene (*suc 2*) from yeast (Johnson *et al.*, 1987). The coding regions of the proteinase inhibitor II gene are indicated by stippled areas; the more intensely stippled areas indicate the extent of the signal peptide. The DNA sequence of the signal peptide is interrupted by one intron (Keil *et al.*, 1986). All constructs were fused to the 35S CaMV promoter and the 3′-end from the octopine synthase gene was added at the *Xmn*I site of the 3′ untranslated region of the *suc 2* gene. Part **B** shows the fusion region between the proteinase inhibitor II part and the invertase gene in more detail. In all three constructs, the authentic invertase coding region started at amino acid number three. In the construct PI-3-INV, the proteinase inhibitor II derived N-terminal region ended three amino acids after the signal peptide cleavage site, whereas the constructs PI-58-INV contained 58 amino acids and the construct PI-113-INV contained 113 amino acids of the mature proteinase inhibitor II protein. The sequence of the region linking the coding regions of the invertase and the proteinase inhibitor II gene is given.

Capped RNA was transcribed *in vitro* using SP6 RNA polymerase. Aliquots were subsequently translated *in vitro* using the reticulocyte lysate system either in the absence or presence of dog pancreas microsomes. The results are summarized in Figure 2. Proteins translated in the presence of microsomal vesicles migrate in SDS gels at higher molecular weights than proteins translated in the absence of vesicles. The latter show a migration behaviour which agrees with the molecular weights calculated for the different fusion polypeptides (compare lanes 1 and 2, 4 and 5 and 6 and 7 in Figure 2). Endoglycosidase H treatment of aliquots translated in the presence of microsomes results in an increase in mobility of the polypeptide, proving that the molecular weight shift is due to attachment of N-linked high-mannose glycans. This indicates that the chimeric proteins have been efficiently core-glycosylated during uptake into the microsomal vesicles (compare lanes 3 and 2 of Figure 2). Thus the *in vitro* translation data show that the chimeric PI-II−invertase protein fusions are translocated across endoplasmic reticulum membranes.

Efficient secretion of PI-II−invertase fusion proteins transiently expressed in protoplasts of Arabidopsis thaliana

The results obtained using *in vitro* translation systems have presented clear evidence that the different chimeric protein fusions are sequestered in microsomal vesicles. To test whether or not this would also hold true for *in vivo* systems, the chimeric genes were inserted into a plant expression cassette, driven by the strong constitutive CaMV 35S promoter, and containing the 3′-end of the octopine synthase gene and introduced into *Arabidopsis thaliana* protoplasts in a transient expression experiment. Thirty-six hours after transformation, both the cells (which start to regenerate cell walls 6 h after cultivation as assayed by calcofluor white staining) and the culture medium were assayed separately for the presence of yeast invertase using protein specific anti-invertase antibodies in immunoblots and an invertase activity stain in SDS gels under semi-native conditions.

The results summarized in Figure 3 show that both immunologically cross-reacting proteins (Figure 3A, lanes 2, 4 and 6) as well as strong invertase activity (Figure 3B, lanes 2, 4 and 6) are present in the concentrated extracellular culture medium whereas only a very weak signal can be detected in the cell extracts (cf. Figure 3A and B, lanes 1, 3 and 5). Mock transformed protoplasts using the expression cassette alone did not give rise to invertase activity or immunologically detectable protein (Figure 3A, lane Col).

Two important conclusions emerge from these results: (i) the chimeric PI-II−invertase fusion proteins are stably expressed in the plant cell giving rise to high invertase activity, and (ii) the proteins are found predominantly in the supernatant of the transiently transformed protoplasts, strongly indicating that due to the absence of any retention signal or vacuolar targeting signal they are efficiently secreted via the default pathway. The molecular weights displayed by the chimeric proteins indicate that they are glycosylated during transit through the endoplasmic reticulum and the Golgi apparatus.

As shown in Figure 3B (lane 7), strong invertase activity is still detectable in the culture fluid of transformed protoplasts which have been embedded in alginate for two weeks and have already formed micro-colonies, strongly indicating that the secretion of the invertase is also occurring efficiently in stably transformed cells (see below).

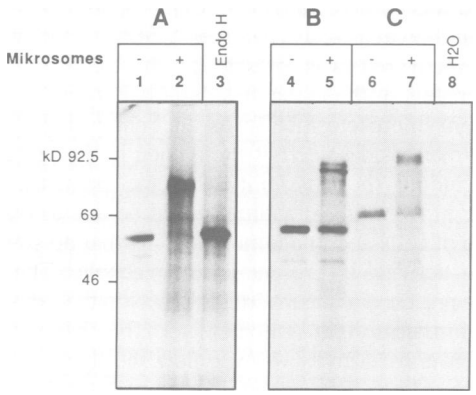

Fig. 2. *In vitro* uptake and glycosylation of different PI-II−INV fusion proteins in microsomal fractions. The three different constructs described in Figure 1 were first modified by replacing the intron-containing genomic sequences of the PI-II gene with cDNA sequences and subsequent fusion behind a SP6 promoter (see Materials and methods). *In vitro*-made RNA obtained from these constructs using SP6 RNA polymerase were, after capping, translated in cell-free translation systems in the absence or presence of microsomal fractions using commercial systems (Promega). *In vitro* translated proteins labelled by the incorporation of [^{35}S]methionine were subsequently subjected to a denaturing polyacrylamide gel electrophoresis and autoradiographed. Part **A** shows the proteins obtained from the PI-3-INV construct in the presence (lane 2) or absence (lane 1) of microsomes. Lane 3 shows the mobility of the translation product obtained in the presence of microsomes after incubation with endoglycosidase H. Parts **B** and **C** show the proteins obtained after *in vitro* translation of the RNA obtained from the constructs PI-58-INV (B) and PI-113-INV (C) in the absence (lanes 4 and 6) or presence (lanes 5 and 7) of microsomal fractions. The position of molecular weight standards is shown in the left-most lane.

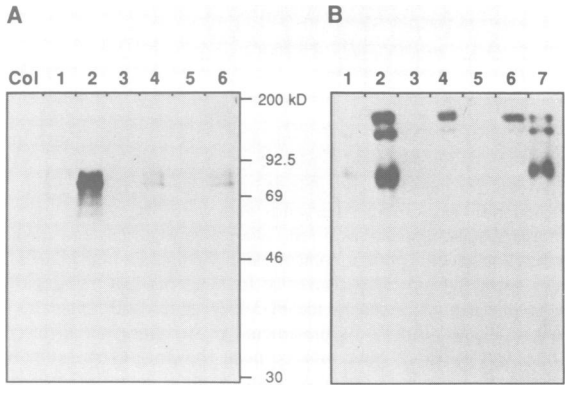

Fig. 3. Transient expression of the different PI−INV fusion proteins in protoplasts of *Arabidopsis thaliana*. Protoplasts isolated from axenically grown *Arabidopsis* plants were transformed using DNA of the three constructs described in Figure 1. After 36 h, protoplasts were pelleted and the proteins present in the supernatant and the pellet were subjected to denaturing (part **A**) or native (part **B**) gel electrophoresis. Proteins were subsequently either transferred to a nitrocellulose membrane and probed with anti-invertase antiserum (part A) or stained *in situ* for invertase activity (part B). In parts A and B, lanes 1, 3 and 5 contain protein present in the pellet and lanes 2, 4 and 6 protein present in the supernatant of constructs PI-3-INV (lanes 1 and 2), PI-58-INV (lanes 3 and 4) and PI-113-INV (lanes 5 and 6). Lane Col in part A contains proteins present in freshly isolated protoplasts of mock-transformed *Arabidopsis thaliana*. Lane 7 in part B contains the supernatant of protoplasts of *Arabidopsis thaliana* embedded in alginate two weeks after transformation. At this stage small microcolonies were visible in the matrix (see Damm *et al.*, 1989).

Expression of the PI-II–invertase protein fusion in stably transformed Arabidopsis thaliana and tobacco plants

The results described in the previous two sections show that the chimeric fusions between N-terminal portions of the PI-II and yeast invertase are efficiently translated into protein and display invertase activity which is predominantly secreted from plant cells.

The three fusions which give different levels of secreted invertase was transferred into *Arabidopsis thaliana* and tobacco plants using *Agrobacterium* mediated gene transfer. Analysis of the regenerated plants revealed the presence of intact non-rearranged genes in the vast majority of the transgenic plants (data not shown). Only those plants containing intact copies of the transferred genes were analyzed further.

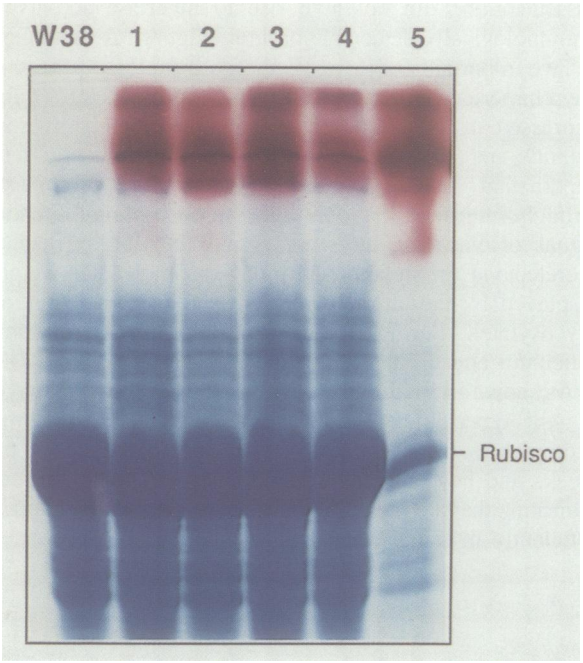

Fig. 4. The PI-3-INV fusion proteins are active in stably transformed plants. Protein extracts from leaves of different tobacco plants (50 μg each except for lane 5) were subjected to a partly denaturing gel electrophoretic separation. Afterwards gels were first stained for invertase activity (appears as a red colour, see Materials and methods) and then for general protein content using Coomassie blue. Lanes 1–4 contain extracts from young leaves of four independent transgenic tobacco plants transformed by the PI-3-INV gene, lane 5 contains proteins extracted from the same amount of starting material (leaf) of an older leaf of a transgenic tobacco plant showing severe symptom development. Lane W38 contains extract from a young leaf of a non-transformed control tobacco plant.

Invertase activity was not detectable in control plants whereas extracts prepared from transgenic plants had high invertase activity (see Figure 4). These data show that the chimeric PI-II–invertase genes are efficiently expressed in stably transformed plants and give rise to an invertase activity which is ~50- to 500-fold higher than that of control plants (see Tables II and III).

In order to determine the relative amounts of invertase in the secreted and intracellular forms in a more quantitative way, protoplasts prepared from leaves of transgenic *Arabidopsis thaliana* plants were cultivated in B5a medium (Damm *et al.*, 1989) at a density of 4×10^5 protoplasts/ml in Petri dishes in the dark.

At time intervals ranging from 3 h up to 68 h after the start of cultivation of the protoplasts, aliquots of this culture were separately assayed for invertase activity contained in the cells (protoplasts) respectively in the extracellular culture fluid. In addition, in order to estimate the possible contamination of the culture medium by broken cells, both the cell pellet and the culture medium were assayed for α-mannosidase, a marker enzyme specific for vacuoles, which upon breakage of the cells would be released into the culture medium from the much more fragile vacuoles. The data obtained are shown as percentage recovery of the total activity and are summarized in Table I.

At time intervals shortly after protoplast isolation, very little invertase activity was detectable in both the (extracellular) culture fluid and the protoplasts. After 18 h of protoplast culture, the total amount of invertase activity had increased by a factor of 9 and a further increase up to ~40-fold was observed after 68 h of protoplast culture. Most significant however is the fact that >96% of the invertase activity recovered was present in the culture medium. Measurements of the α-mannosidase activity as a vacuolar marker enzyme performed in parallel show that 80–90% of the α-mannosidase activity remained in the protoplasts, thus excluding the possibility that the presence of the invertase in the culture medium might be due to excessive breakage of cells and in addition proving that no significant invertase activity is present in the vacuole. The nearly exclusive presence of the invertase activity in the extracellular space is furthermore in agreement with the observation that the total invertase activity was very low shortly after isolation of the protoplasts. Due to the liberation of single cells and digestion of the cell wall during protoplast isolation, invertase present in the apoplast would be lost during washing steps

Table I. Distribution of invertase activity in the extracellular (culture medium) and intracellular spaces of transgenic *Arabidopsis thaliana* cells

Cultivation time (hours)	Total	Invertase activity (Arbitrary units)				α-Mannosidase activity (Arbitrary units)	
		Extracellular	%	Intracellular	%	Extracellular	Intracellular
3	7	3	43	4	57	2	35
6	18	8	44	11	56	3	39
18	57	44	77	13	23	4	37
30	91	79	87	12	13	8	28
68	274	264	96	10	4	6	36

Protoplasts were prepared from 4 week old plants stably transformed with the shortest chimeric proteinase inhibitor II–invertase gene (see Materials and methods). Purified protoplasts were subsequently incubated in B5a medium at a density of 4×10^5 protoplasts/ml and the distribution of invertase activity and α-mannosidase activity determined in the extracellular fluid (culture medium) or cells as described in Materials and methods.

included in the protoplast isolation procedure (Damm and Willmitzer, 1988).

The nearly quantitative recovery of the invertase activity in the extracellular culture medium, together with the observation that the chimeric PI-II−invertase protein is highly glycosylated (data not shown and Figure 3) excludes the possibility of any significant amount of the invertase being present in the cytoplasm or the vacuolar compartment in these transgenic plants, but rather proves that within the limits of detection, this chimeric invertase is almost exclusively secreted from the transformed cell.

Constitutive secretion of yeast invertase into the apoplastic space of transgenic tobacco plants leads to dramatic changes in their phenotype

When transgenic tobacco plants expressing the yeast-derived invertase in the extracellular space were transferred into soil and subsequently grown further in the greenhouse, they displayed several rather dramatic changes in their phenotype (see Figure 5A−D). In general, tobacco plants expressing invertase showed a significantly reduced height (see Figure 5A for an example). Considerable variation was observed between different transformants, the smallest tobacco plants reaching a height of only 5 cm and other transformants almost reaching the height of a wild-type plant (i.e. ~150 cm). This depression in size was due to reduced internodal distance and not to a reduction in the number of leaves forming.

A closer inspection of the leaves showed another phenotype. Thus in older leaves bleached regions appeared in the interveinal tissue which in some cases became necrotic, resulting in a phenotype reminiscent of senescence phenomena, whereas in other transgenic plants, leaves bleached but no necrotic reactions were visible (see Figure 5B for different examples).

More significant is the fact that this phenotype appeared in a precise order which follows a developmental pattern: Firstly necrosis and/or bleaching always started at the tip or rim of the leaf moving towards the base during leaf expansion. Secondly, symptoms only developed in older leaves, whereas the 3−4 youngest leaves were always visually indistinguishable from the corresponding leaves of a wild-type plant (see Figure 5D).

A third phenotype specific for invertase-expressing transgenic plants is a significant reduction in root formation. As shown in Figure 5C, root formation was suppressed to different extents in independent transformants.

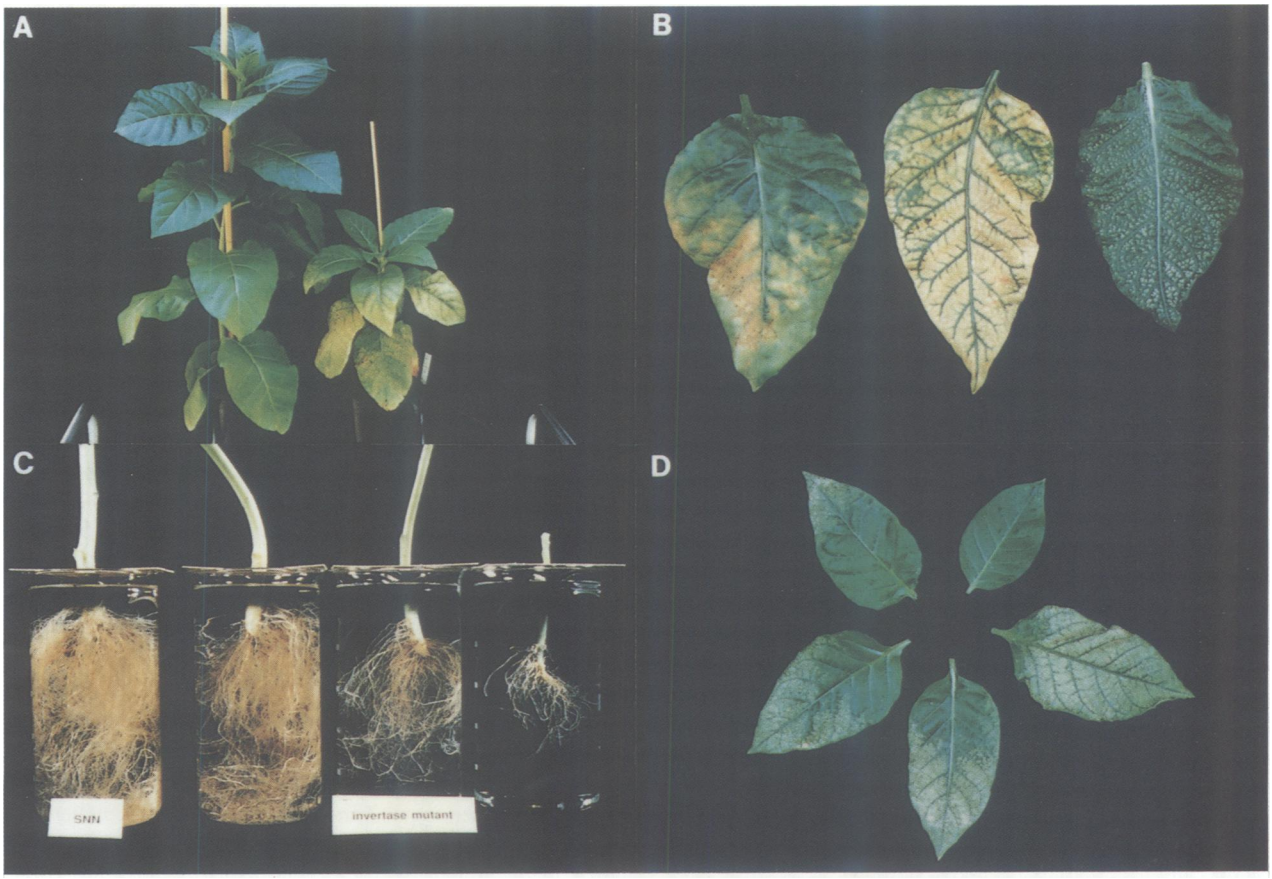

Fig. 5. Influence of the constitutive expression of an apoplastic invertase on development and habitus of transgenic tobacco plants. **A**. Stunted growth of transgenic tobacco plant (right) expressing medium levels of the yeast invertase as compared with transgenic tobacco plant where the expression of the invertase was below detection level (left). **B**. Spectrum of symptoms observed in leaves of three different invertase-expressing transgenic tobacco plants. **C**. Impaired root development of three transgenic tobacco plants expressing increasing levels of invertase (increasing from left to right) as compared with a non-transformed control plant (SNN). **D**. Development of symptoms associated with expression of invertase in leaves of transgenic tobacco plants follows a distinct developmental pattern. Anticlockwise arrangement of the 5th to 9th leaves (counted from the top) of a transgenic tobacco plant expressing high levels of invertase.

Table II. Invertase, Chl, sugars and photosynthesis in different tobacco transformants

Genotype	Chl	Invertase	Sugars[a]			Photosynthesis[a]
	mg m^{-2}	μmol m^{-2} s^{-1}	Glc	Fru	Sucr	μmol O_2 m^{-2} s^{-1}
			mmol hexose m^{-2}			
Wild-type	264	0.42	1.1	0.8	1.2	19.5
1-92	121	12.2	4.8	3.8	4.5	14.0
1-95	174	8.5	1.6	3.4	2.3	18.0
10 leaf edge	211	55.0	5.4	2.8	5.6	18.0
leaf middle	342	17.7	1.4	1.2	2.1	28.9

[a]Measured after 6 h illumination.

Constitutive secretion of yeast invertase in the apoplastic space of transgenic Arabidopsis plants does not lead to dramatic changes with respect to phenotype except excessive formation of anthocyanins and/or discolouration in older leaves

When transgenic *Arabidopsis thaliana* plants expressing different levels of the yeast invertase were transferred into soil and grown further in the greenhouse, no dramatic differences with respect to growth rate or final size were observed between transformed and wild-type plants. The only differences emerging were either the appearance of dark red pigments in older leaves of transformed plants indicating anthocyanin formation, which appeared in leaves of invertase-expressing *Arabidopsis* plants significantly more often than in wild-type plants, or a discolouration of the older leaves which again started at the tip of the leaves (data not shown).

Apoplastic expression of invertase leads to accumulation of carbohydrates and inhibition of photosynthesis

Analysis of transgenic tobacco plants expressing invertase in the apoplast. As described above, young leaves of transgenic tobacco plants expressing invertase were green and lost chlorophyll as they matured. The phenotype differed in details depending on the transformant. Two transformants displaying either a mosaic-like phenotype (transformant nos. I-92 and I-95) (see Figure 5B, right leaf) or displaying distinct bleached and green areas (transformant no. 10) (see Figure 5B, left leaf and Figure 5D, all leaves) were analysed in more detail.

In the case of mosaic leaves, considerable amounts of starch accumulated in the pale areas. The area close to the veins had low levels of starch with the exception of the veins themselves which contained considerable levels of starch (data not shown). These leaves had, overall, a decreased chlorophyll (Chl) content (Table II). They also had increased soluble sugars, especially the free hexoses, and the overall rate of photosynthesis was lower (Table II).

In the case of tobacco transformants showing distinct bleached and green areas, iodine staining of leaves revealed high levels of starch in many of the pale areas but little or no starch in the green areas (data not shown). The pale sectors contained 4- to 5-fold more glucose, fructose and sucrose than the dark green sections. Photosynthesis was slightly inhibited in the pale areas, and increased in the green areas, compared to the wild-type (Table II, see also below for more data).

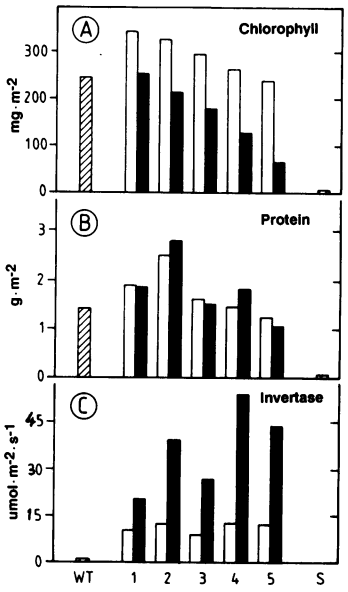

Fig. 6. Levels of Chl, protein and invertase in successive leaves of tobacco transformant 10. The green (□) and pale (■) sections of five successive leaves on the plant are shown. For comparison the values in mature wild-type leaves (WT,▨) and fully senesced leaves of the wild-type plant (S,▥) are shown on the left and right sides, respectively. **A**: Chl; **B**: protein; **C**: invertase.

The experiment described in Table II was carried out with young leaves in which the phenotype had just started to develop. In a further experiment, leaves were sequentially sampled down a tobacco plant to investigate the relation between invertase activity, carbohydrate accumulation, and photosynthesis in more detail as the phenotype developed. These experiments were carried out with a transformant showing distinct areas of bleached and green areas allowing uniform (with respect to visual phenotype) leaf discs to be removed from each tissue. For comparison, leaf discs were also taken from a mature leaf and a fully senesced leaf on a wild-type plant. The changes of Chl (Figure 6A) quantify the visible phenotype. The green leaf sectors contained more Chl than the wild-type, while there was a progressive loss of Chl in the pale sectors as the leaf matured. The protein levels were similar in both leaf sectors (Figure 6B).

Alkaline invertase activity was very low (<1 μmol m^{-2} s^{-1}) in the leaves from wild-type plants, and did not change during leaf development (data not shown). In the transgenic plant, there was already considerable invertase activity (12 ± 3 μmol m^{-2} s^{-1}) in the youngest sink leaves, which did not yet show any signs of bleaching. The green areas

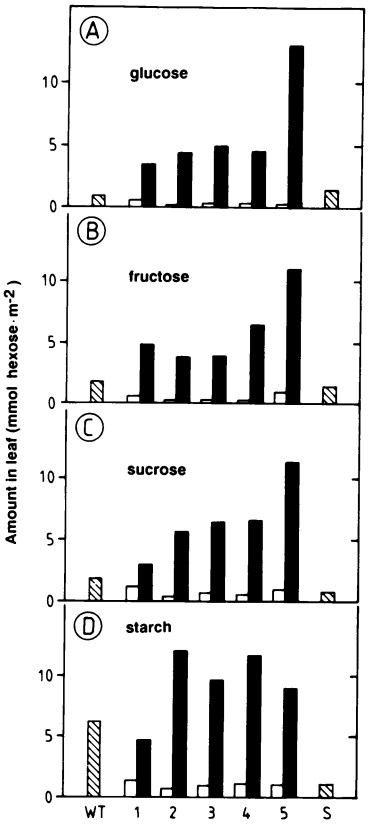

Fig. 7. Levels of carbohydrate in successive leaves on transformant 10. **A**: glucose; **B**: fructose; **C**: sucrose; **D**: starch. Leaves were harvested after 6 h illumination. The symbols are as in Figure 6.

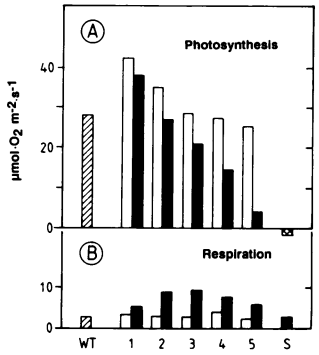

Fig. 8. Rates of photosynthesis and respiration in successive leaves of transformant 10. **A**. Photosynthesis measured in saturating light and CO_2 in an O_2 electrode. **B**. Respiration. The symbols are as in Figure 6.

of the older leaves also contained invertase activity in this range (Figure 6C). They contained low levels of glucose, fructose, sucrose and starch (Figure 7A–D) after 6 h of illumination. These green areas had high rates of photosynthesis and low rates of respiration (Figure 8A,B). The leaf sectors which bleached show an additional four-fold increase in invertase activity (Figure 6C), which might be either a consequence of the general loss of protein in these leaves or due to increased levels of expression of the transgene in these areas. They also showed a progressive increase in the levels of glucose, fructose and sucrose and had a high starch content after 6 h of illumination (see Figure 7A–D). At an advanced stage, the pale sectors of the leaf contained 10- to 20-fold more carbohydrate than

Table III. Turnover of carbohydrate in tobacco leaves

	Wild-type		Transformant 10			
			Green Sector		Pale Sector	
	D	L	D	L	D	L
	(mmol hexose m^{-2})					
Glucose	1.1	1.1	0.6	1.0	9.8	10.9
Fructose	0.9	0.9	0.8	1.3	8.1	9.8
Sucrose	1.3	2.2	1.8	3.1	4.5	11.7
Starch	1.6	2.6	1.5	10.4	8.3	10.3

D = measured after 12 h darkness.
L = measured after 12 h illumination.

green sectors on the same leaf. The rate of photosynthesis in these pale areas decreased progressively as the leaf aged and the rate of respiration increased 2- to 3-fold (Figure 8A,B). In the older leaves, the net rate of photosynthesis in the bleached sector was six-fold lower than the rate of photosynthesis in the immediately adjacent green sectors of the same leaf.

Senescing leaves in the wild-type (shown on the right side of each panel in Figures 6–8) showed a very different response. There was a dramatic loss of protein (Figure 6B). They did not accumulate sucrose or starch, there was only a small increase in hexoses (Figure 7A–D) and respiration did not increase (Figure 8B).

We next investigated carbohydrate turnover in these tobacco plants (Table III). The high level of carbohydrate in the bleached leaf sectors only decreased slightly during the night. In contrast, in the green areas of the same leaf, considerable amounts of carbohydrate, especially starch, accumulated during the day, and were almost completely remobilized during the night.

Analysis of transgenic Arabidopsis plants expressing invertase in the apoplast. As described above, young leaves of transgenic invertase-expressing *Arabidopsis* plants were green. As they matured, the leaves began to turn yellow and red at the tip and edges with the discolouration eventually speading over the entire leaf.

Two different transformants (A-14 and A-19) were investigated. Leaves were harvested when they were still fully green ('young'), or after their tip and edges had started to discolour ('middle') or after they fully discoloured ('old'). The visual phenotype was reflected in the Chl content as in the case of transgenic tobacco plants (Table IV). The young green leaves of transgenic plants actually contained more chlorophyll than the wild-type; this has also already been observed for the invertase expressing tobacco plants (see Figure 6A).

Invertase activity was already 50-fold above the wild-type in the young green leaves of both transformants. Invertase increased another 20–40% (on an area basis) as the leaves discoloured. Protein decreases as the leaves matured.

The young green leaves from transformed plants contained only slightly higher levels of soluble sugars than the wild-type. Discoloured leaves contained 5- to 15-fold higher levels of free hexoses, and 4-fold higher levels of sucrose than the wild-type. The level of starch was already high in the wild-type, and did not increase much further in the transgenic plants. The carbohydrate levels in Table IV were measured after 8 h light period. Similar results were obtained at the end of the photoperiod (i.e. 16 h) except that the carbo-

Table IV. Invertase activity, Chlorophyll, protein, sugars, photosynthesis and respiration in two different *Arabidopsis* transformants.

Genotype	Leaf	Chl	Invertase	Protein	Sugars[a]			Starch[a]	Photosynthesis[a]	Respiration[b]
		mg m^{-2}	μmol m^{-2} s^{-1}	g m^{-2}	Glc	Fru	Sucr	mmol hexose m^{-2}	μmol O_2 m^{-2} s^{-1}	μmol O_2 m^{-2} s^{-1}
					mmol hexose m^{-2}					
Wild-type		157	0.13	2.68	0.4	0.6	0.7	9.3	11.5	1.8
A-14	young	191	7.0	5.81	1.1	2.0	1.7	12.7	15	2.0
	middle	126	9.1	2.22	2.4	4.2	1.7	11.6	11	2.2
	old	49	11.1	2.45	5.1	7.0	2.9	13.2	1	1.5
A-19	young	212	6.8	5.05	0.6	0.9	1.5	13.5	18	2.0
	middle	168	6.6	1.96	4.7	6.9	3.5	12.5	12.5	1.7
	old	74	8.5	1.70	5.6	9.8	2.6	12.5	1.8	1.8

[a]after 8 h light.
[b]after 16 h dark.

hydrate levels in the wild-type were ~2-fold higher (data not shown).

Young green leaves from the transgenic plants had a 25–50% higher rate of photosynthesis than leaves from the wild-type. The accumulation of carbohydrate in older leaves was accompanied by an almost total inhibition of photosynthesis. The rate of respiration did not change significantly.

Discussion

Despite the central importance of sink–source relationships not only for plant development but also for the yield of crop plants, little is known about major determinants controlling this relationship.

More specifically, there are no mutants available which could serve as a defined source to study this relationship. This could be either due to the fact that mutations in sink–source relationships are lethal because of their central importance for plant development, or due to the fact that because photoassimilate partitioning is a highly complex process, mutants would display pleiotropic effects and thus not be recognized as mutants being affected in sink–source relationships.

In order to overcome this problem two strategies are feasible following a genetic approach: (i) genes or gene products could be identified which are specific indicators for either sink or source tissues. In the case that a clear relationship existed between the expression of these genes and the state of the tissue being a sink or a source, they could be used as indicators allowing the identification of mutants impaired in sink–source functions irrespective of pleiotropic effects exerted by the individual mutation. (ii) Genes or gene products which, in view of their biochemical properties, represent candidates possibly involved in determining sink–source relationships could be introduced into plants using reversed genetics and expressed in a specific way to try to interfere with normal sink–source related processes. Here we describe data obtained via the second approach.

As outlined in the Introduction, in this series of experiments we specifically intended to interfere with the export of sucrose from source leaves. The reason for this experiment was on the one hand to study the effects of such a fairly drastic change of sink–source relationships on plant development and growth, on the other hand to use a genetic approach to test experimentally the hypothesis that photosynthesis can be sink-limited. This latter question is of obvious importance to future analysis and understanding of sink–source relations in higher plants.

To achieve this we constructed transgenic tobacco and *Arabidopsis* plants expressing chimeric genes composed of signal sequences from a vacuolar protein linked to sequences encoding the mature yeast invertase and introduced into a plant expression cassette driven by the 35 S CaMV promoter. The signal peptides were expected to ensure uptake of the protein fusions into the ER of the plant cells. Due to the absence of positive sorting information, the fusions were expected to become secreted by default as is known for mammals and yeast, whereas targeting of vacuolar proteins seems to require positive sorting information (Dorel *et al.*, 1989; Voelker *et al.*, 1989).

Data presented here show that all chimeric protein fusions with N-terminal portions of the potato PI-II linked to yeast invertase are efficiently secreted by transformed tobacco and *Arabidopsis* cells. Although the longest fusion lacks only ten C-terminal amino acids of the PI-II coding sequence, yeast invertase did not accumulate inside the cells. Moreover, none of the protein fusions was detected by immunological methods in vacuoles isolated from leaf mesophyll protoplasts of stably transformed plants, whereas PI-II was (data not shown).

As a result of the expression of invertase in the apoplast, several severe changes in the phenotype of transgenic tobacco plants were observed, such as stunted growth, impaired root formation and bleached areas and necrotic lesions on older leaves. The two first phenomena, i.e. impaired root formation and reduced (stunted) growth, can probably be linked directly to the fact that expression of an apoplastic invertase in source leaves of tobacco should impair the ability of these leaves to export sucrose efficiently which would be reflected in the development of the different sink organs (such as stem and root). Another sink organ where one might expect changes are seeds. Although changes in the germination frequency of transgenic tobacco seeds were observed, the effect was not as striking as in the case of the roots and needs further investigation.

Concerning the last phenomenon mentioned, i.e. bleaching and development of necrotic lesions on older leaves, it is important to recall that this phenomenon follows a strict developmental pattern: every plant analysed showed a vertical progression in phenotype, i.e. the older the leaves, the more they were affected, whereas the 3–4 youngest leaves were phenotypically unaffected. Within each single leaf a developmental pattern is again followed: the symptoms

first appeared at the leaf tip and/or rims from where they move gradually towards the leaf basis. Finally, a gradient can be observed between different types of leaf tissue: phenomena first become apparent in the mesophyll tissue and subsequently spread towards the smaller veins and finally also appear around the larger veins.

In tobacco, as in most other higher plant species, sucrose is the preferred if not exclusive compound used in long-distance transport of photoassimilates from source to sink tissues. Young small leaves depend for their development both on photoassimilates formed by the leaf itself and on photoassimilates supplied by older source leaves. In the transgenic tobacco plants, young leaves do not display any symptoms, demonstrating that the expression of yeast invertase is of no harm to these sink leaves.

Upon further development, the sink leaf develops into a source leaf starting at the leaf tip (see Turgeon, 1989). The criterion for becoming a source leaf is defined by a net export of sucrose from the photosynthetically active tissues. In order to export the sucrose, the mesophyll cells have to load sucrose into the sieve elements via the companion cells. Whereas it is broadly accepted that the sucrose moves symplastically within the mesophyll cells, the mechanism of loading of the companion cells is a matter of debate. Both symplastic routes as well as routes involving an apoplastic loading step have been proposed (Giaquinta, 1983). The phenotype observed with the transgenic invertase-expressing tobacco plants can, however, only be explained if an apoplastic loading step is involved. Sucrose present in the apoplast is at least partly split by yeast invertase yielding the two hexoses, fructose and glucose. Hexoses are not taken up into the phloem. Thus only two possibilities remain. The hexoses could accumulate in the apoplast, leading to an increase in osmotic potential and thus to plasmolysis of adjacent mesophyll cells which would lead to death of the tissue. This phenomenon would therefore resemble at least partly the normal senescence process occuring in petals where plasmolysis has been observed (Matile and Winkenbach, 1971). A second mechanism would infer that the hexoses are efficiently taken up by the mesophyll cells, where they probably become phosphorylated yielding hexose phosphates which could re-enter the pathway for sucrose synthesis, resulting in a futile cycle (Foyer, 1987; Huber, 1989). As one consequence these cells would run out of free phosphate, probably resulting in a reduction of triosephosphate export from the chloroplast via the triosephosphate/phosphate antiport (Flügge and Heldt, 1984) leading to photoinhibition and finally to pigment bleaching in the leaves due to photodestruction. Both mechanisms are obviously not mutually exclusive and could also act in a synergistic fashion.

Whatever the exact mechanism, both models imply a membrane transport step into the apoplast, thus strongly supporting an apoplastic loading of the phloem.

The biochemical data described in Figures 6–8 and Tables II–IV are in agreement with the assumption of photosynthesis being feed-back-inhibited by sink demand.

Young leaves are sinks; they retain their photosynthate and use it to support their own growth. In agreement, even though these young leaves contain high activities of invertase, they do not accumulate carbohydrate. In both *Arabidopsis* and tobacco, the youngest leaves also contain more Chl and have higher rates of photosynthesis than the wild-type. This would be in accordance with the photosynthesis being sink-limited as now, due to the lower supply of these leaves with sucrose from source leaves, there is a higher demand for sucrose synthesized within the sink leaf itself. As a leaf matures, it turns into a source leaf and exports sucrose to the rest of the plant. If export is blocked by the apoplastic invertase, carbohydrates would be expected to accumulate in the leaf, which is in agreement with the experimental findings.

The loss of chlorophyll and photosynthetic capacity observed for older leaves is clearly linked with this accumulation of carbohydrate. The causal relation is demonstrated most clearly in the tobacco transformant no. 10, where closely adjacent segments of the same leaf develop in a strikingly different way. Part of the leaf contained low levels of carbohydrate, and turned this carbohydrate over on a daily basis. The Chl content and rates of photosynthesis in these sectors were as high as, or even higher than those in the wild-type. Other sectors accumulated high levels of glucose, fructose, sucrose and starch. In these sectors there was a progressive loss of Chl, photosynthesis was inhibited and respiration increased. These plants provide a clear demonstration that photosynthetic metabolism is inhibited when carbohydrates accumulate in the leaf.

The starch levels in the bleached leaf areas were often only slightly higher than those in the wild-type, at least at the end of the day (Table III). This might suggest that the inhibition of photosynthesis is probably related to increased levels of soluble sugars, rather than starch. There was an increase of sucrose, glucose and fructose. Presumably, after the sucrose is split, the free hexoses are reabsorbed, rephosphorylated and used to resynthesize sucrose. The relative levels of sucrose, glucose and fructose will reflect the balance between invertase activity and these retrieval mechanisms. We cannot decide from our data alone which of these soluble sugars is responsible for the inhibition of photosynthesis. However it is apparent that a 2- to 4-fold increase above the levels found in the wild-type leads to a very considerable inhibition of photosynthesis, which again would be in agreement with the assumption that photosynthesis is sink-limited although other explanations independent of a natural feed-back system (e.g. prolonged exposure to abnormal osmotic stress) cannot be excluded completely.

Changes at the biochemical level have been observed for both transgenic *Arabidopsis thaliana* and tobacco plants. Strikingly, however, symptom development in *Arabidopsis* plants growing in soil was much less severe when compared with tobacco plants, the main symptom observed being the increased anthocyanin formation in lower leaves and/or the discolouration of older leaves. These symptoms can both be explained by photoinhibition. In this case, the role of anthocyanins would be to capture excessive light which the plant is no longer able to use in photosynthesis. Thus a similar mechanism to that discussed for the tobacco plants would be inferred. However, the striking difference between the development of tobacco and *Arabidopsis* plants expressing apoplastic invertase remains.

In conclusion, we have expressed yeast invertase in the apoplastic space of transgenic tobacco and *Arabidopsis* plants under the control of a constitutive promoter. In the case of tobacco, dramatic changes in the development and habitus of transgenic plants are observed which are clearly linked to sink–source interactions.

At the biochemical level, expression of the apoplastic invertase leads to a decreased sucrose export and accumula-

tion of carbohydrate in the leaf. As a result, photosynthesis is inhibited and, in tobacco, respiration increases. These results demonstrate that sink–source relationships can be altered by introducing and expressing a single gene in transgenic plants, which provides a novel and well characterized experimental system to study physiological questions at the molecular level.

Materials and methods

Plants, bacterial strains and media

Nicotiana tabacum L. variety Samsun NN was obtained through 'Vereinigte Saatzuchten', Ebsdorf, FRG. *Arabidopsis thaliana* L. variety Columbia C24 was kindly provided by J.P.Hernalsteens (Vrije Universiteit Brussel, Belgium). Plants in tissue culture were grown under a 16 h light/8 h dark regime (~3000 lux, 50% relative humidity) on Murashige and Skoog media (Murashige and Skoog, 1962) containing 2% sucrose (2MS) at 28°C in the case of tobacco and at 22°C on half concentrated 2MS medium (AM) in the case of *Arabidopsis* plants (Schmidt and Willmitzer, 1988).

Plants used for biochemical analysis were grown in a 3:1 mixture of John Innes no. 1 compost and sand, and were watered daily with a solution containing 5 mM KNO_3, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 mM Fe–EDTA, 70 μM H_3BO_3, 14 μM $NiCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.3 μM NaMoCl, 10 μM NaCl, 0.01 μM $CoCl_2$. The plants were grown in a 12 h light/12 h dark cycle (light 300 μmol m^{-2} s^{-1}, temperature 20°C) in individual pots (diameter 12 cm, depth, 12 cm).

Escherichia coli strains DH5a (Bethesda Research Laboratories, Gaithersburg, MD, USA), BMH71-18 (Messing *et al.*, 1977) and GJ23 (Van Haute *et al.*, 1983) were cultivated and handled using standard techniques (Maniatis *et al.*, 1982). *Agrobacterium tumefaciens* strain C58C1 containing either pGV3850kan (Jones *et al.*, 1987) or pGV2260 (Deblaere *et al.*, 1985) were cultivated in YEB medium (Vervliet *et al.*, 1975).

Reagents

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Ingelheim, FRG) and New England Biolabs (Danvers MA, USA). Endoglycosidase H and b-fructosidase (yeast invertase) were from Boehringer Mannheim. m7GpppG cap structure analogue was from New England Biolabs. Cellulose Onozuka R-10 and Macerozyme R-10 were from Serva (Heidelberg, FRG). SP6 *in vitro* transcription kit, multiprime labelling kit, [α-^{32}P]dUTP and [α-^{32}P]dCTP (>3000 Ci/mmol), [^{35}S]methionine (>1000 Ci/mmol), Rainbow™-marker and ^{14}C-methylated protein standards were obtained from Amersham Buchler (Braunschweig, FRG). Rabbit reticulocyte lysate minus methionine (nuclease treated), goat anti-rabbit IgG (Fc) Alkaline Phosphatase conjugate and pGEM™ vectors were from Promega Corp. (Madison, WI, USA). 'Protein-Assay', and other reagents for SDS–PAGE were purchased from BioRad (Richmond, CA, USA). 2,3,5-trimethyltetrazoliumchloride and all other reagents were obtained through Sigma Chemical Co. (St Louis MO, USA) or Merck (Darmstadt, FRG).

Dog pancreas microsomes were kindly provided by B.Dobberstein (EMBL, Heidelberg, FRG). Protein specific anti-yeast invertase antisera were generous gifts of R.Schekman (UC Berkley, CA, USA) and L.Lehle (Universität Regensburg, FRG).

Anti-invertase specific antibodies were purified from the crude sera by affinity chromatography using b-fructosidase (Boehringer Mannheim) coupled to CNBr-activated Sepharose (according to the protocol recommended by the supplier, LKB-Pharmacia, Bromma, Sweden).

Plasmid constructions

PI-3-INV, PI-58-INV and PI-113-INV fusions were assembled in pUC18 (Yanisch-Perron *et al.*, 1985). For the two longer fusions, PI-II genomic clone E32 (Keil *et al.*, 1986) was cut either with *ScaI*/*BanII* or *ScaI*/*SphI*, generating 570 bp and 400 bp fragments, respectively. After trimming the 3′-protruding ends, both fragments were separately inserted into the *SmaI* site of pUC18. For the shortest fusion we took advantage of a deletion clone ending shortly behind the signal sequence processing site in the PI-II coding sequence (M.Keil, personal communication). Upon digestion with *ScaI* and *EcoRI*, the resulting 230 bp fragment was introduced into the *SmaI* site of pUC18. For generating the short translational fusion with yeast invertase, the *HindIII* site upstream of the *suc2* gene of pSEYC306 (Johnson *et al.*, 1987) was filled in and a *BamHI* linker (octamer) was introduced, thereby deleting the *PstI* and *SalI* sites in the pSEYC306 polylinker. From the resulting plasmid (pSEYC306-8) the 2.2 kb *BamHI–XmnI suc2* fragment was inserted 3′ of the 230 bp PI-II fragment in pUC18, generating the short PI-II–yeast invertase fusion PI-3-INV. To generate the two longer fusions, a *HindIII* linker was introduced downstream of the *suc2* gene after filling in the remaining *SalI*-site in pSEYC306-8. The *suc2* gene was excised as a 2.4 kb *HindIII* fragment and subcloned into pKM109-3 (Reiss *et al.*, 1984). From a clone with the desired orientation (5′ end of *suc2* gene adjacent to *BamHI* site in pKM109-3), the 2.2 kb *BamHI–XmnI suc2* fragment was excised and inserted 3′ of the 400 bp and the 570 bp PI-II fragments in pUC18, giving rise to constructs PI-58-INV and PI-113-INV, respectively. The constructs were introduced as *Asp718–SphI* fragments into a plant expression cassette, containing the 35S promoter of CaMV and the polyadenylation signal of the octopine synthase gene as well as into pGEM™ vectors (Promega) following removal of the intron from the PI-II signal sequence and its replacement with sequences derived from a PI-II cDNA clone (Sánchez-Serrano *et al.*, 1986).

In vitro transcription and translation

Preparative amounts of capped RNA were synthesized from the constructs in pGEM™ vectors by incubating 5 μg of linearized template DNA in 50 μl SP6 transcription mix for 60 min at 40°C using an SP6 kit from Amersham. Aliquots of 2 μl were directly translated in the rabbit reticulocyte lysate system (Promega) including 0.5 μCi (>1000 Ci/mM) [^{35}S]methoinine. For ER uptake studies, samples also included 2 μl aliquots of dog pancreas microsomes or blank mix. Aliquots of 5 μl were withdrawn from the 30 μl translation mixtures, suspended in SDS loading buffer, boiled for 5 min, loaded onto 10% SDS–polyacrylamide gels and analysed by fluorography after electrophoresis. Endoglycosidase H treatment of translation mixtures were performed as described by Trimble and Maley (1984).

Transient expression in Arabidopsis protoplasts

Isolation, transformation and cultivation of *Arabidopsis* protoplasts was essentially as described by Damm *et al.* (1989). Routinely, 15 μg plasmid DNA of the pGSC1704 constructs were used to transform 10^6 protoplasts. After transformation, protoplasts were cultivated for 36 h in the dark at 24°C. Cells were pelleted (5 min RT at 100 × *g*) and the cleared culture medium (3 ml) was concentrated in 'Centricon 30' microconcentrator devices (Amicon, Danvers, MA, USA) by centrifugation at 5000 × *g* and 4°C in a fixed angle rotor (Beckman JA20) yielding ~50 μl in volume. Cells were lysed by multiple freeze–thaw cycles in liquid nitrogen. Equivalent volumes of cells (equivalent to 5 × 10^5) and concentrated culture medium were suspended in loading buffer either containing 2% SDS, boiled for 5 min and analysed by immunoblots or suspended in gel loading buffer without SDS, separated by electrophoresis and stained for invertase activity.

Protoplasts prepared from transgenic *Arabidopsis* plants were embedded in alginate gels and cultivated according to the protocol described by Damm and Willmitzer (1988). The culture medium was exchanged weekly, concentrated and analysed for the presence of secreted invertase as described above.

Immunoblots

Following separation in 10 % SDS–polyacrylamide gels (Laemmli, 1970) proteins were transferred onto nitrocellulose membranes using a semi-dry electroblotting apparatus ('Multiphor II', LKB Bromma, Sweden). Saturation of the filter was for 30 min in TBS (20 mM Tris–HCl pH 7.5, 500 mM NaCl) containing 2% newborn calf serum (Sigma). Incubation with affinity purified anti-invertase antibodies (1:2000 dilution in TBST, i.e. TBS containing 0.1% Tween-20) was for 2 h at RT followed by three successive wash steps for 10 min in TBS, TBST and TBS. Incubation with secondary antibodies (alkaline phosphatase conjugated goat-anti-rabbit IgGs, 1:7500 in TBST) was for 60 min at RT. Following three successive wash steps as above, development of the filter was in AP buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) supplied with 0.1 mg/ml f.c. nitro blue tetrazolium (NBT) and 0.05 mg/ml f.c. 5-bromo-4-chloro-indolylphosphate (BCIP).

Invertase activity stain

Detection of yeast invertase activity in 10% SDS–polyacrylamide gels under semi-native conditions was based on the method of Gabriel and Wang (1969) described in Carlson *et al.* (1981). Cell extracts were suspended in loading buffer without SDS and subjected to SDS–PAGE without prior heating (Laemmli, 1970). After electrophoresis, gels were briefly rinsed in distilled water and incubated in acidic sucrose solution (0.1 M sucrose, 0.1 M NaOAc pH 5.0) for 60 min at 37°C. Following three wash steps in distilled water for 5 min, gels were developed by boiling in 0.5 M NaOH containing 0.1% 2,3,5-triphenyltetrazoliumchloride, giving rise to pink bands at positions of invertase activity.

Transfer of constructs into Agrobacterium strains

Mobilization of cointegrate vectors from *E.coli* into *Agrobacterium* strain C58C1:pGV3850kan was as described by Van Haute *et al.* (1983).

Constructs in binary vectors were directly transformed into *Agrobacterium* strain C58C1:pGV2260 as described by Höfgen and Willmitzer (1988).

Stable transformation of plants

Plant transformation using the *Agrobacterium tumefaciens* leaf disc technique for tobacco was as described by Rosahl *et al.*, (1987) and for *Arabidopsis* as described by Schmidt and Willmitzer (1988).

Analysis of transgenic plants

Total protein was extracted from plant tissue as described by Racusen and Foote (1980). Protein concentrations were determined with 'Protein Assay' (BioRad) and BSA as a standard. Unless otherwise indicated, routinely 30 μg of total protein were subjected to SDS−PAGE and analysed by immunoblotting or invertase activity stains.

Measurements of the α-mannosidase activity were performed essentially as described (Sonnewald *et al.*, 1990).

Analysis of the distribution of invertase and α-mannosidase activity in the extracellular and intracellular compartments of Arabidopsis plants transformed with the shortest PI-II−invertase fusion. Protoplasts were prepared from 4 week old plants as described (Damm *et al.*, 1989) and cultivated at a density of 4×10^5 protoplasts/ml in the dark in Petri dishes in B5a medium (Damm *et al.*, 1989). After different time intervals, aliquots were taken off and protoplasts pelleted as described (Damm *et al.*, 1989). The supernatant was transferred into a fresh tube and centrifuged for another 5 min at 2500 *g* to get rid of cellular debris. The protoplast/cell pellet was washed once in W5, frozen and proteins extracted in 200 μl buffer I (50 mM HEPES pH 7.0; 2 mM Na bisulphite, 0.1% SDS and 2 μg/ml of leupeptin, pepstatin and antipain) by vortexing in the presence of glass beads. After pelleting of the cellular debris, extracted proteins were assayed for invertase activity as described.

The supernatant of the first centrifugation containing the culture fluid was concentrated using 'Centricon 30' tubes. Proteins were precipitated with ammonium sulphate (75%), washed, resuspended in the same volume (200 μl) of buffer I as the pelleted cells and assayed for invertase activity as described. An aliquot of the proteins isolated from the extracellular fluid and the cells respectively was assayed in parallel for α-mannosidase activity.

Measurement of photosynthesis and respiration. For both *Arabidopsis* and tobacco plants, eight leaf dics (diameter 6.5 mm) were removed and placed in a leaf disc O_2 electrode (Hansatech, Kings Lynn, UK). Illumination was 570 μmol m^{-2} s^{-1} and saturating CO_2 was supplied from 400 μl 2 M carbonate−bicarbonate buffer, pH 9.3, applied to a felt mat in the base of the electrode (Quick *et al.*, 1984). The temperature was 20°C. After 10 min (photosynthesis) or 15 min (respiration) the leaf discs were removed and frozen in liquid N_2 under continuing illumination or darkness, respectively. The leaf discs were homogenized to a fine powder under liquid N_2 and aliquots were then removed for assay of enzymes and carbohydrates.

For quantitative measurement of alkaline invertase, powder equivalent to 5−10 μg fresh weight (FW) was removed with a precooled spatula, and was rapidly extracted by vortexing it in 0.3 ml buffer (50 mM HEPES−KOH, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EGTA, 5 mM dithiothreitol, 2 mM benzamidine, 2 mM aminocaproic acid, 0.5 mM PMSF, 0.1% (v/v) Triton X-100 and 10% (v/v) glycerol), homogenizing it in a glass homogenizer, and then snap freezing 60 μl aliquots in liquid N_2. Alkaline invertase was measured by incubating 25 μl extract with 75 μl buffer (27 mM KOH−HEPES pH 7.5, 133 mM sucrose) for 3 h at 25°C, heating (95°C, 4 min), and then assaying enzymatically for glucose and fructose (see below).

To measure sugars and starch, the remaining extract (60−70 μg FW) was extracted in 800 μl 10% (v/v) $HClO_4$, for 15 min at 0°C and centrifuged (3 min, 18 000 *g*). The supernatant was immediately neutralized with 5 M KOH, 1 M triethanolamine, recentrifuged and the supernatant used for enzymatic analysis of glucose, fructose and sucrose as described (Stitt *et al.*, 1983). The sediment was dissolved in 5 ml 80% ethanol and centrifuged (10 min, 2000 *g*). The supernatant was taken for pheophytin and protein measurement (see below). The sediment was resuspended in 0.5 ml H_2O, and washed by centrifugation until the pH was >6. It was then autoclaved and assayed for starch as in Stitt *et al.* (1978).

Chlorophyll was measured in enzyme extracts by adding 1 ml ethanol to 60−120 μl extract, centrifuging (5 min, 18 000 *g*), and measuring the absorbance at 652 nm. Chlorophyll in the extract for sugar analysis was measured in the resuspended pellet from the $HClO_4$ extraction (see above). The chlorophyll was converted to pheophytin in this pellet. Absorbance was measured at 652 nm, using an empirically measured extinction coefficient (1 mg pheophytin/ml = 30.2 E). The total Chl in the leaf sample was calculated by summing the Chl in the extracts for enzyme determination and sugar determination. Protein was measured according to Bradford (1976) in aliquots from the resuspended $HClO_4$ sediment. The protein in the total leaf sample was estimated by summing both.

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